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1) Survey of gene amplifications during prostate cancer progression by high-throughput fluorescence in situ hybridization on tissue microarrays

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AUTHOR ADDRESS: Cancer Genet. Branch, Natl. Human Genome Res. Inst., NIH, 49 Convent Dr., MSC 4470, Room 4A24, Bethesda, MD 20892-4470, USA\*\*USA

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Department of Pediatrics, Oregon Health Sciences University, Portland 97201, USA.

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3) \*\*\*Glycotyping of prostate specific antigen.

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Thank you

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# Characterization of Insulin-Like Growth Factor-Binding Protein-Related Protein-1 in Prostate Cells\*

VIVIAN HWA, CINDY TOMASINI-SPRENGER, ABEL LÓPEZ BERMEJO,  
RON G. ROSENFELD, AND STEVEN R. PLYMATE

Department of Pediatrics, Oregon Health Sciences University (V.H., A.L.B., R.G.R.), Portland, Oregon 97201; and the Geriatric Research, Education, and Clinical Center, Veterans Affairs Health Care System Puget Sound (C.T.-S., S.R.P.), Tacoma, Washington 98493

## ABSTRACT

Insulin-like growth factor-binding protein-related protein-1 (IGFBP-rP1; also known as Mac25, TAF, and PSF) is a member of the IGFBP superfamily. It is a cysteine-rich protein that shares structural and functional similarities with the conventional IGFBPs. *In situ* hybridization of prostate tissue sections show intense IGFBP-rP1 messenger ribonucleic acid (mRNA) expression in normal stroma and glandular epithelium. There was a significant loss of detectable IGFBP-rP1 mRNA in metastatic prostate tissue. IGFBP-rP1 mRNA (Northern blots) and protein (immunoblots) were detectable in primary cultures of prostatic stromal and epithelial cells as well as in the

immortalized nonmalignant prostatic human epithelial cells, P69, and in the P69 metastatic subline, M12. IGFBP-rP1 expression was not detectable in the prostatic cancer cell lines PC-3, DU145, and LNCaP. IGFBP-rP1 expression was regulated in P69 cells but not in M12 cells. Protein and mRNA expression was up-regulated by IGF-I, transforming growth factor- $\beta$ , and retinoic acid. The observations that IGFBP-rP1 expression is significantly diminished in prostate tumorigenesis and is regulated in nonmalignant prostate cells suggest IGFBP-rP1 is important in normal prostatic cell growth. (*J Clin Endocrinol Metab* 83: 4355–4362, 1998)

THE PROLIFERATION of human prostatic cells is controlled by the complex regulation of many hormones and growth factors, including insulin-like growth factors (IGFs) (1). IGFs, of which there are two (IGF-I and IGF-II), are peptides that are structurally related to insulin. Both IGF peptides exert growth-stimulating effects on prostate cells (1–4), mainly through interactions with the type I IGF receptors (IGF-IR) found on cell surfaces. Perturbation of IGF levels and IGF availability have been hypothesized to contribute to malignant prostatic cell growth, a hypothesis supported by recent clinical studies. In one investigation, men with increased serum levels of IGF-I were found to have a 4-fold higher probability of contracting prostate cancer (5). In another study, young males with acromegaly, a disease characterized by abnormally high levels of circulating IGFs, were found to have enlarged prostates (6).

In biological fluids, IGFs are normally sequestered by IGF-binding proteins (IGFBPs), of which there are six, designated IGFBP-1 to -6 (7–10). As IGFs have higher binding affinities for IGFBPs than for the IGF-IR, IGFBPs are important in the modulation of IGF biological activity. The effect can be an inhibition (11, 12) or an enhancement of IGF-IGF-IR interactions (13). In addition to these IGF-dependent actions of IGFBPs, IGFBPs have important biological actions indepen-

dent of their abilities to bind IGFs. The IGF-independent effects of IGFBPs can be antiproliferative (14, 15) or growth stimulatory (16).

The IGFBP family has recently been expanded to include a group of additional cysteine-rich proteins that are involved in regulating cell growth (17–19). These IGFBP-related proteins (IGFBP-rPs) share structural features with the conventional IGFBPs, IGFBP-1 to -6 (20). For the conventional IGFBPs, a striking shared feature is the conservation of critical cysteines, clustered at the N-terminus third (12 cysteines) and the C-terminus third (6 cysteines) of the proteins (7). It has been hypothesized that the N- and C-termini are independent domains that together are responsible for the high affinity IGF binding characteristic of IGFBPs (21). The IGFBP-rPs contain the N-terminal domain of the IGFBPs, but their C-terminal domains have clearly diverged (17, 20, 22, 23). In addition to structural similarities, there are functional similarities between the IGFBP-rPs and IGFBPs. Two of the IGFBP-rPs, IGFBP-rP1, Mac25 (22), and IGFBP-rP2, CTGF (23), are able to bind IGF-I, although with a 20- to 100-fold reduced affinity compared to that of IGFBP-3 (18, 19). The existence of cysteine-rich proteins with conserved N-terminus domains and demonstrable abilities to bind IGFs, albeit with lower affinities than the conventional IGFBPs, has led to the proposal of an IGFBP superfamily, subdivided into high affinity IGF binders (IGFBP-1 to -6) and low affinity IGF binders (IGFBP-rPs) (19). The ability of these low affinity IGF binders to modulate IGF bioactivity *in vivo* is not known, and only scant data are available on their IGF-independent actions.

The conventional IGFBPs in the prostate have been well characterized, whereas virtually nothing is known regarding prostate IGFBP-rPs. IGFBP-2 to -6 have been detected in

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Address all correspondence and requests for reprints to: Dr. Vivian Hwa, Department of Pediatrics, NRC5, Oregon Health Sciences University, 3181 SW Sam Jackson Park Road, Portland, Oregon 97201.

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stromal and epithelial prostate cells as well as in prostatic epithelial cell lines (1, 24–30). The pattern of detectable IGFBPs is altered in malignant prostatic cells (31, 32). The significance of changes in IGFBP levels in malignancy has yet to be determined, but probably includes altered modulation of IGF bioactivity (4, 33, 34) as well as biological effects independent of IGFs (35–37).

Of the IGFBP-rPs, IGFBP-rP1 (Mac25) messenger ribonucleic acid (mRNA) has been detected in the prostate (18). The complementary DNA (cDNA) for Mac25 (22) was originally cloned from leptomeningial cells by differential display. The *mac25* cDNA was found to be preferentially expressed in normal leptomeningial and mammary epithelial cells compared to their counterpart tumor cells (22, 38) and to be up-regulated in senescent human mammary epithelial cells (38). These results suggested that *mac25* played a role in growth-regulating pathways that are abrogated in meningiomas and breast carcinoma. The same apparent protein and cDNA have been isolated from human bladder carcinoma cells [tumor-derived adhesion factor (TAF)] (39) and from human diploid fibroblast cells [prostacyclin-stimulating factor (PSF)] (40). Functionally, TAF at high concentrations ( $>1 \mu\text{g/mL}$ ) appears to promote cell adhesion of cancer cells and to stimulate growth of mouse fibroblasts (41, 42); PSF was shown to stimulate prostacyclin synthesis in endothelial cells. Oh *et al.* (18) synthesized that the Mac25 protein (which they redesignated IGFBP-7) in a baculovirus system and demonstrated that it can bind IGF as well as insulin; similar results were obtained by Yamanka *et al.*, using purified TAF protein (21). A Northern blot showing tissue distribution of IGFBP-rP1 (Mac25/TAF/PSF/IGFBP-7) mRNA indicated an abundance of the mRNA in normal prostate and a distinct decrease of mRNA levels in prostate cancer cells (18). This result supports the hypothesis that IGFBP-rP1 is involved in regulating cell growth.

As IGFBP-rP1 is potentially important in the regulation of normal prostate cell growth, we studied the mRNA and protein expression of IGFBP-rP1 in both normal and malignant prostate cells and demonstrated that in immortalized nonmalignant epithelial cells, P69 cells (43), IGFBP-rP1 expression is regulated by IGF-I, transforming growth factor- $\beta$  (TGF $\beta$ ), and retinoic acid (RA).

## Materials and Methods

### Materials

F-12 nutrient mixture (Ham's) powder, epidermal growth factor (EGF), dexamethasone, all-*trans*-retinoic acid, and the additive ITS (insulin, transferrin, selenium) were purchased from Sigma Chemical Co. (St. Louis, MO). RPMI 1640, HEPES, fungizone, and gentamicin were obtained from Life Technologies (Grand Island, NY). IGF-I was a gift from Eli Lilly & Co. (Indianapolis, IN). TGF $\beta$ 1 was purchased from Austral Biologicals (San Ramon, CA). Bovine pituitary extract was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). FBS was obtained from HyClone Laboratories, Inc. (Logan, UT). Nitrocellulose and electrophoresis reagents were purchased from Bio-Rad Laboratories, Inc. (Richmond, CA); nylon membranes (GeneScreen) were obtained from New England Nuclear (Boston, MA). Horseradish peroxidase-linked donkey antirabbit IgG and enhanced chemiluminescence detection reagents were purchased from Amersham (Arlington Heights, IL). Polyclonal antibody against IGFBP-rP1 (IGFBP-7) (18) was generated in rabbits (44).

### In situ hybridization

Prostate tissues were obtained from patients undergoing radical prostatectomies and processed for *in situ* hybridization as described previously (26, 27). The cDNA used for *in situ* hybridization was a 0.88-kb *SspI-XbaI* IGFBP-rP1 cDNA fragment cloned in pBluescript SK<sup>+</sup> (Stratagene, La Jolla, CA). Antisense mRNA was prepared from the linearized plasmid from the T7 promoter. Specificity of the hybridization was determined by the use of duplicate slides that were hybridized with <sup>35</sup>S-labeled RNA probe and a 100-fold excess of unlabeled RNA. Specificity of this probe is also indicated by hybridization to a single 1.1-kb mRNA species on Northern blots of total cytoplasmic RNA from primary cultures of human prostate epithelial cells and prostate stromal cells.

### Cell culture

Biopsies of prostate tissue from the central and peripheral zones were obtained during radical prostatectomies. The tissue samples were digested, and the epithelial and stromal cells were separated (28). The primary epithelial cells are composed of predominantly basal epithelial cells; however, a central portion of the culture reacts with an antibody to prostate-specific antigen as well as to an antibody to cytokeratin-8, suggesting a luminal component. Primary epithelial cells were maintained in HEPES/F-12 medium supplemented with 10 ng/mL EGF, 0.1  $\mu\text{mol/L}$  dexamethasone, 5 ng/mL selenium, bovine pituitary extract, fungizone, and gentamicin, whereas stromal cells were cultured in HEPES/F-12 medium supplemented with fungizone, gentamicin, and 5% FBS. Both lines were maintained at 37 C under 5% CO<sub>2</sub>.

The derivation of the P69 and M12 cell lines has been previously described (43, 45). P69 cells are simian virus 40 T antigen-immortalized, normal human prostate epithelial cells that are poorly tumorigenic. M12 cells are a metastatic subline of P69 cells generated by serial passage through athymic mice. Both cell lines were cultured in RPMI 1640 medium supplemented with 10 ng/mL EGF, 0.1  $\mu\text{mol/L}$  dexamethasone, 5  $\mu\text{g/mL}$  insulin, 5  $\mu\text{g/mL}$  transferrin, 5 ng/mL selenium, fungizone, and gentamicin at 37 C under 5% CO<sub>2</sub>.

All cells used in these experiments were mycoplasma free as determined by the Mycoplasma PCR Primer Set (Stratagene).

### Growth factor treatment studies

All cell lines mentioned above were grown to 80% confluence in 100-mm tissue culture dishes and treated with various doses of IGF-I (0–100 ng/mL), RA (0, 10<sup>-7</sup>, 10<sup>-9</sup>, 10<sup>-11</sup>, and 10<sup>-13</sup> mol/L), or TGF $\beta$  (0, 1, and 5 ng/mL) in RPMI 1640 supplemented with 5  $\mu\text{g/mL}$  transferrin and 5 ng/mL selenium. After 24 h of treatment, medium and total cytoplasmic RNA were collected for Western immunoblots (see *Western immunoblot analysis*) and Northern blots (see *RNA analysis*), respectively. All experiments were repeated in triplicate.

### Western immunoblot analysis of IGFBP-rP1 expression

Media taken from both treated and untreated (control) cells were normalized based on cell counts and concentrated by filtration through nitrocellulose. After concentration, proteins were redissolved in 23  $\mu\text{L}$  denaturing SDS sample buffer [0.5 mol/L Tris (pH 6.8), 1% SDS, 10% glycerol, and 8 mol/L urea] and boiled for 10 min. For studies specifically involving P69 cells, conditioned media (CM) were not concentrated, as IGFBP-rP1 was readily detectable. Samples were electrophoresed on 12% SDS-polyacrylamide gels, then electroblotted onto nitrocellulose. Western blots were incubated with IGFBP-rP1 antiserum at a 1:3000 dilution in Tris-buffered saline-Tween-20 (0.1%) overnight at 4 C. Blots were washed with Tris-buffered saline-Tween-20 and then incubated for 1 h at 22 C with a 1:2500 dilution of horseradish peroxidase-linked antirabbit IgG secondary antibody. IGFBP-rP1 was detected with ECL chemiluminescence reagents according to the manufacturer's protocol.

### RNA analysis

Total cytoplasmic RNA was isolated from cells using RNeasy (Qiagen, Inc., Chatsworth, CA). Eight to 10  $\mu\text{g}$  of each RNA preparation were electrophoresed on a 1.2% agarose-2.2 mol/L formaldehyde gel, trans-



ferred overnight onto a nylon membrane (GeneScreen, DuPont, Wilmington, DE) using  $10 \times$  SSC (standard saline citrate) as the transfer solution, and cross-linked to the membrane by UV irradiation in a Stratalinker 1800 (Stratagene). The Northern blots were then probed with a 660-bp *EcoRI-SmaI* fragment of the IGFBP-rP1 cDNA (18), which was radiolabeled ( $1 \times 10^9$  dpm/ $\mu$ g) with [ $\alpha$ - $^{32}$ P]deoxy-CTP (New England Nuclear-DuPont; SA, 3000 Ci/mmol) using a random priming kit (Prime-a-Gene, Promega Corp., Madison, WI). Northern blots were hybridized overnight at 42 C in 50% formamide,  $5 \times$  SSC,  $10 \times$  Denhardt's solution, 1% SDS, and 100  $\mu$ g/mL sheared denatured herring sperm DNA; in some cases, hybridization was performed using Rapid-Hyb buffer (Amersham) and according to the manufacturer's instructions. Blots were then washed for 30 min in  $2 \times$  SSC at room temperature, for 30 min in  $2 \times$  SSC-0.1% SDS at room temperature, and stringently washed at 55 C in  $0.2 \times$  SSC-0.1% SDS for 10–20 min. Blots were exposed to Kodak XAR film or to Kodak Biomax MS film (Eastman Kodak Co., Rochester, NY) for 1–4 days at  $-70$  C using one intensifying screen. Membranes were then stripped in SDS for 10–30 min and reprobbed with actin or 18S, which acted as a loading control for the RNA samples. An image analyzer equipped with MCID version 4.2 software (Imaging Research, Inc., St. Catharines, Canada) was used to quantify the resulting bands.

In addition to the blots prepared from the growth factor studies,

Northern blots prepared with either polyadenylated RNA or total RNA isolated from microdissections of normal and tumorigenic prostate tissue were obtained from Dr. Peter Nelson (University of Washington, Seattle, WA). These blots were probed, incubated, washed, and analyzed as described above.

## Results

### *In situ hybridization of prostate tissue*

*In situ* hybridization experiments using  $^{35}$ S-labeled antisense cDNA to IGFBP-rP1 were performed on prostate tissue sections, normal and malignant, to determine the expression of IGFBP-rP1. Normal tissue from 27 prostate glands and lymph nodes from 2 men in which prostate cancer had metastasized were examined, and representatives are shown in Fig. 1. In normal prostate tissue, intense labeling of IGFBP-rP1 message was detected in stromal areas as well as in the glandular epithelium that surrounds the lumen (Fig. 1, A and B). Note that there is a clear increase in grain intensity from luminal epithelial cells to basal epithelial cells, suggesting

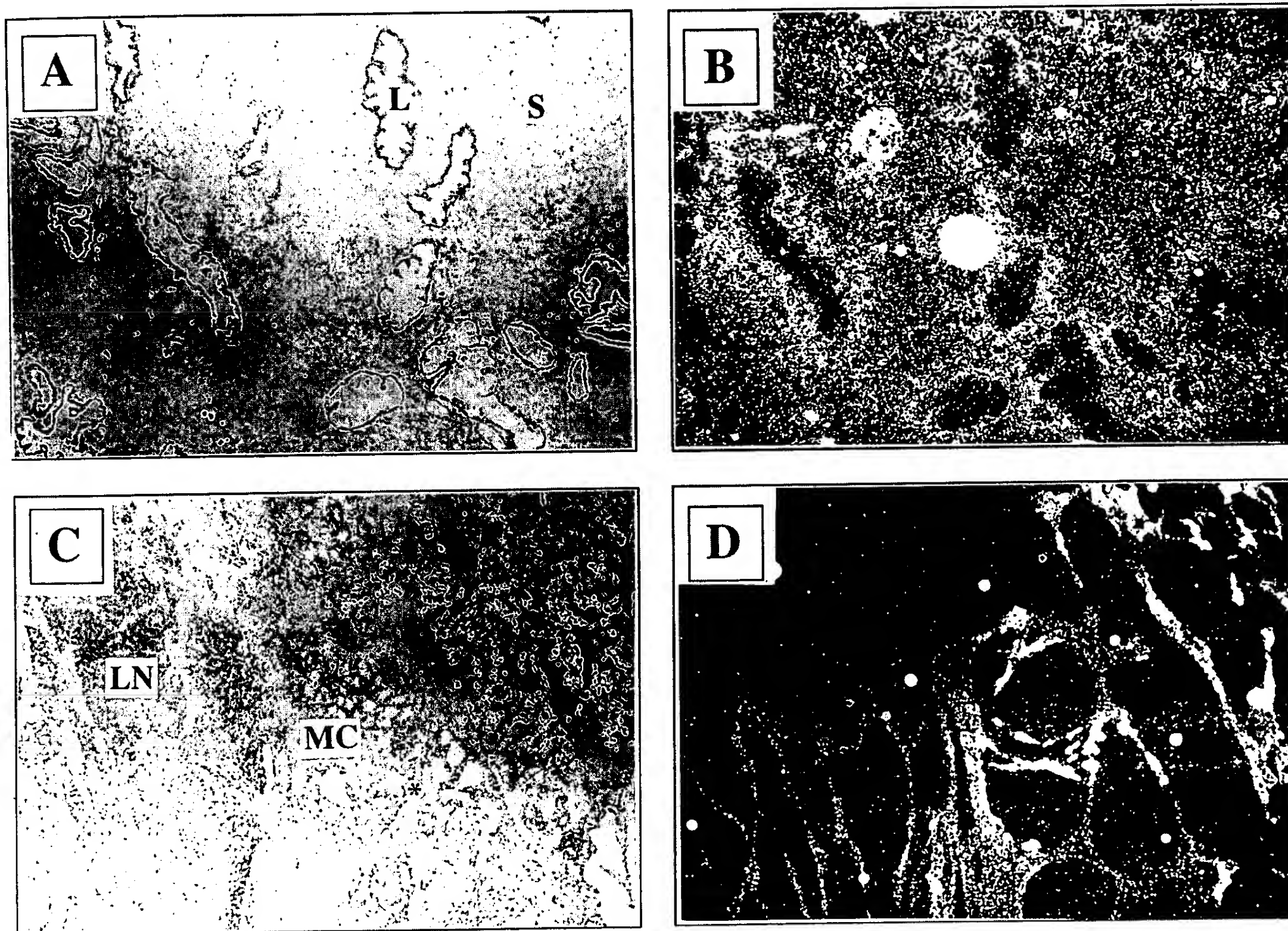


FIG. 1. *In situ* hybridizations of prostate tissue sections. *In situ* hybridization has been performed with a  $^{35}$ S-labeled antisense cDNA to IGFBP-rP1 (see *Materials and Methods*). A, Lightfield ( $\times 200$ ) photomicrograph of normal human prostate tissue. S, Stroma; L, lumen. B, Darkfield ( $\times 200$ ) photomicrograph of the same section shown in A. Note the intense grain density in the stromal areas but also the outline of grains in the glandular epithelium. C, Lightfield ( $\times 200$ ) photomicrograph of prostate cancer that has metastasized to a lymph node. LN, Lymph node; MC, metastasized cancer. D, Darkfield ( $\times 200$ ) photomicrograph of the same section shown in C. Note the loss of  $^{35}$ S grains in the metastatic lymph node and the presence of IGFBP-rP1 mRNA in both the lymph node stroma and cancer tissue stroma (indicated by an asterisk). To test for specificity, 100-fold more unlabeled IGFBP-rP1 cDNA than  $^{35}$ S-labeled cDNA was added to the hybridization mixture. There was no specific labeling above background (not shown).

that IGFBP-rP1 mRNA is produced by both luminal and basal epithelial cells. In contrast, malignant prostate tissues demonstrate a dramatic loss in detectable IGFBP-rP1 mRNA, as shown in tissue sections of prostate cancer metastasized to a lymph node (Fig. 1, C and D). Note that the lymph node itself produces minimal IGFBP-rP1 mRNA. In the metastasized tissue, the prominent prostate epithelial cells appear to express very little IGFBP-rP1, although IGFBP-rP1 mRNA was still readily detectable in stromal areas. The labeling of IGFBP-rP1 mRNA was specific, as unlabeled IGFBP-rP1 cDNA (100-fold excess) reduced signals to background levels (data not shown). The marked decrease in IGFBP-rP1 mRNA expression in malignant prostate cells compared to normal cells was further supported by Northern blots of mRNA extracted from microdissections of human prostate epithelial cells (Fig. 2). There was a progressive loss of detectable IGFBP-rP1 mRNA going from normal cells to malignant prostate epithelial cells.

#### Expression of IGFBP-rP1 in cell lines

Although IGFBP-rP1 mRNA is present and readily detectable in prostate, it is not clear whether IGFBP-rP1 protein is transcribed from these transcripts. The rabbit polyclonal antibody generated against baculovirus-purified IGFBP-rP1 (anti-IGFBP-7) (44) has been employed for immunoblotting and immunoprecipitations to demonstrate the presence of IGFBP-rP1 protein in biological fluids and CM of various cell lines (44). Therefore, to further characterize IGFBP-rP1 mRNA and protein in the prostate, IGFBP-rP1 expression in primary cultures of normal epithelial and stromal prostate cells as well as in simian virus 40 T-antigen transformed epithelial cells, P69 (43), and its tumorigenic and metastatic subline, M12 (45), were analyzed. Northern blots of total RNA extracted from these cells indicate detectable IGFBP-rP1 mRNA of approximately 1.1 kb in all cell lines, with the most abundant message detected in primary stromal cells (Fig. 3B) and the least abundant in M12 cells.

Immunoblot analysis of CM from the prostate cell lines shows an approximately 31- to 32-kDa protein that is immunoreactive with our anti-IGFBP-rP1 antibody (Fig. 3A).

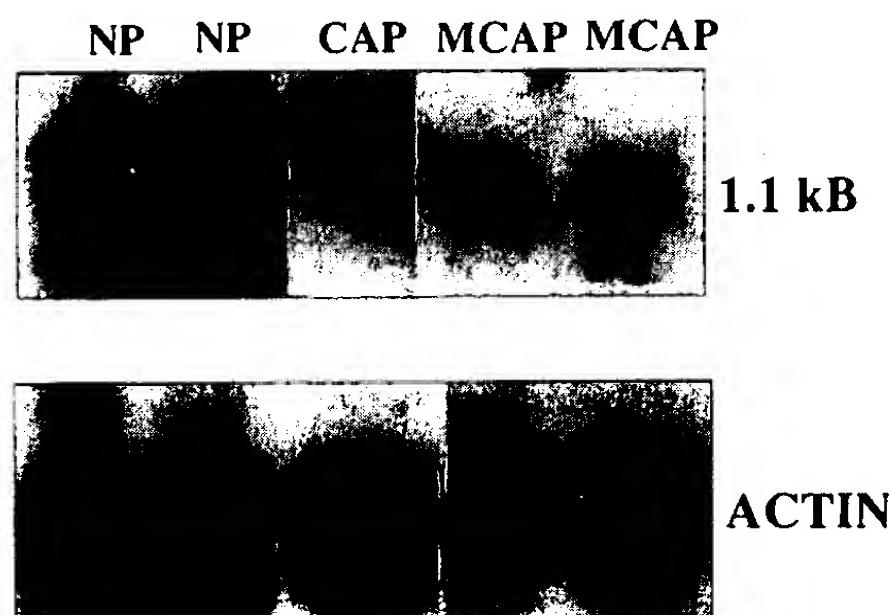


FIG. 2. Northern blot of mRNA isolated from microdissected human prostate epithelial cells. Note the increased signal for the IGFBP-rP1 mRNA in the normal prostate epithelial cells (NP), decreasing signal in the malignant epithelial cells from within the prostate (CAP), and the more marked decrease in the metastatic prostate epithelial cells (MCAP). Loading of mRNA is compared to an actin control.

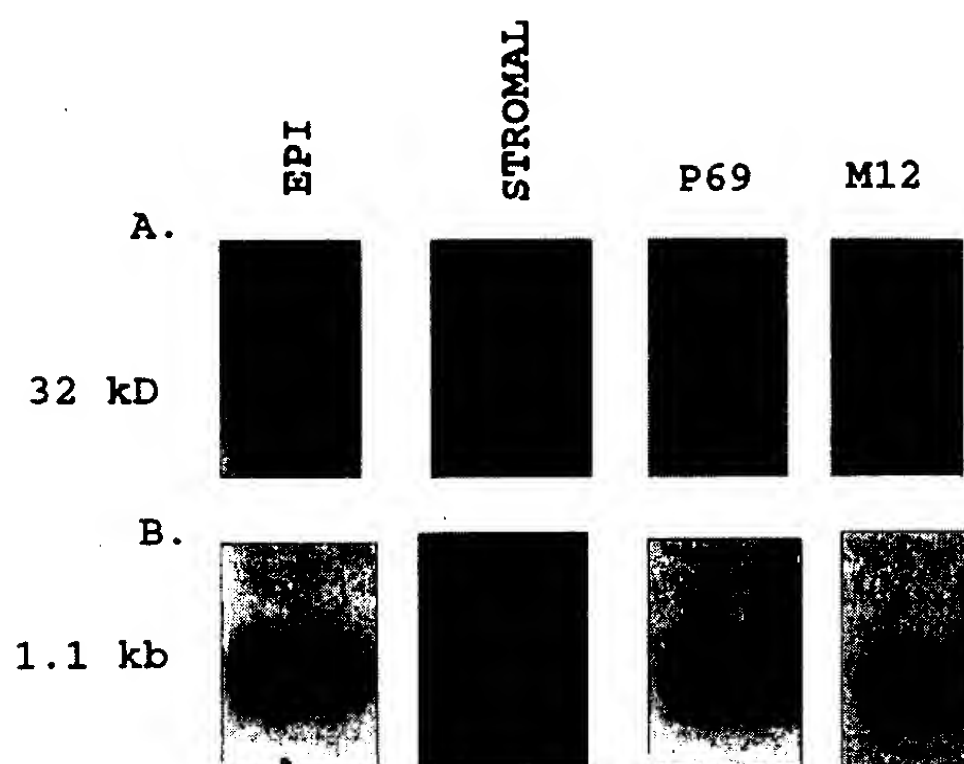


FIG. 3. Detection of IGFBP-rP1 expression in prostate cell cultures. Primary cultures of prostate epithelial cells (EPI) and stromal cells as well as P69 cells and its subline, M12 cells, were grown as indicated in *Materials and Methods*. CM and total mRNA were collected and subjected to immunoblot and Northern blot analysis. A, Immunoblots of CM from cultured cells. The size of IGFBP-rP1 is indicated on the left (32 kDa). B, Northern blot showing detectable IGFBP-rP1 mRNA (1.1 kb).

The size of the protein detected is consistent with the size of IGFBP-rP1 that was detected in biological fluids and in CM from the breast cancer cell line Hs578T (44). The concentrations of IGFBP-rP1 detected in the CM from the prostate cell lines suggest that M12 expresses the least amount of IGFBP-rP1, with stromal cells expressing the highest concentrations. The IGFBP-rP1 protein concentration detected in CM from stromal cells reflects the high level of IGFBP-rP1 mRNA detected.

Expression of IGFBP-rP1 mRNA and protein was also investigated in the well established prostate cancer cell lines, PC-3, LNCaP, and DU145 cells. In serum-free medium, IGFBP-rP1 mRNA was detectable by Northern blots, but IGFBP-rP1 protein was not detectable in CM by either immunoblots or immunoprecipitation of CM from <sup>35</sup>S-Met-labeled cells (data not shown).

#### IGFBP-rP1 expression is regulated in P69 cells

In normal mammary epithelial cells, *mac25* (IGFBP-rP1) is regulated by RA (38). To determine whether IGFBP-rP1 is regulated in prostate cells, we investigated the effects of various growth factors (IGF-I, TGF $\beta$ , and RA) on expression of IGFBP-rP1 in P69 cells and its subline, M12 cells. P69 cells are responsive to the growth stimulatory effects of IGFs, whereas M12 cells are considerably less responsive, probably due to an 80% decrease in IGF-I receptors per cell compared to those in P69 cells (46). Epithelial cells are known to be inhibited by growth factors such as TGF $\beta$  and RA. Hence, examining the effects of these three factors on IGFBP-rP1 expression might provide clues for the role(s) of IGFBP-rP1 in prostate cell proliferation.

IGFBP-rP1 in P69 cells is regulated by all three growth factors tested (Figs. 4-6). In contrast, IGFBP-rP1 in M12 was not regulated at the concentrations of growth factors tested (data not shown). IGF-I up-regulated IGFBP-rP1 in P69 cells in a dose-dependent manner at both mRNA and protein



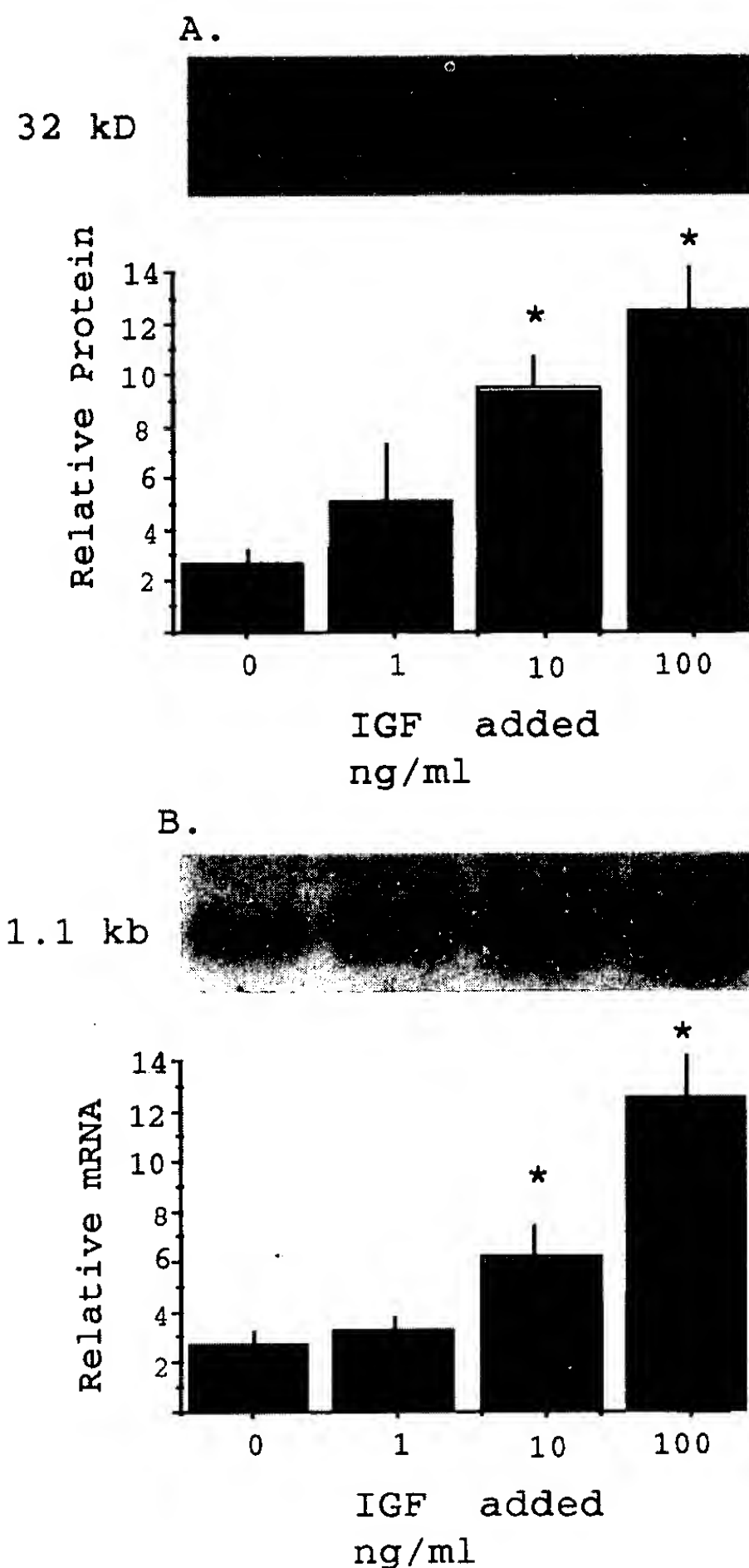


FIG. 4. Regulation of IGFBP-rP1 expression by IGF-I in P69 cells. P69 cells were treated with increasing doses of IGF-I, and CM and total mRNA were collected after 24 h. A, Immunoblot analysis of IGFBP-rP1 expression. Bar graphs indicate relative amounts of IGFBP-rP1 protein, based on densitometric readings of immunoblots. B, Northern blot analysis of IGFBP-rP1 expression. Bar graphs indicate relative concentrations of IGFBP-rP1 mRNA based on densitometric readings of Northern blots. The asterisk indicates statistical significance ( $P < 0.05$ , by Student's  $t$  test).

levels (Fig. 4). IGFBP-rP1 protein showed increases of 3.5-fold at 10 ng/mL and 5-fold at 100 ng/mL (Fig. 4A), and mRNA levels were increased 2.5- and 5-fold, respectively, compared to those in untreated cells. Western ligand blot of CM also showed that at these concentrations of IGF-I, some of the IGFBPs detectable in P69 cells, in particular IGFBP-2, -3, and -4 (46), were also significantly increased (data not shown).

Both RA and TGF $\beta$  increased IGFBP-rP1 expression at the message and protein levels. The effect of RA on IGFBP-rP1 expression was modest, with a 2-fold increase in IGFBP-rP1

protein expression (at  $10^{-7}$  mol/L RA), as determined by Western immunoblot, and a 2-fold increase in IGFBP-rP1 mRNA (Fig. 5). Western ligand blots of the same CM indicated that RA, even at 1  $\mu$ mol/L (data not shown), did not change the levels of the other IGFBPs in P69 cells. TGF $\beta$  up-regulated IGFBP-rP1 mRNA and protein dose dependently and significantly, although the increases were also modest (Fig. 6). IGFBP-rP1 mRNA was increased 2.5-fold in cells treated with 5 ng/mL TGF $\beta$ , and IGFBP-rP1 protein was increased 2-fold. Of the IGFBPs, IGFBP-3 protein was up-regulated by TGF $\beta$  concomitantly with IGFBP-rP1 (data not shown).

### Discussion

The biological functions of IGFBP-rP1 [also known as Mac25 (22), TAF (39), PSF (40), and IGFBP-7 (18)] have yet to be defined, but have been reported to be associated with senescence (38), stimulation of prostacyclin production in endothelial cells (40), and enhanced adhesion of cancer cells (39). In leptomeningeal cells and mammary epithelial cells, it was noted that IGFBP-rP1 (Mac25) mRNA was significantly decreased in malignant cells compared to that in normal cells. Our results are consistent with these observations, as *in situ* hybridizations of prostate tissue sections as well as mRNA extracted from microdissections indicated that expression of IGFBP-rP1 mRNA was significantly decreased in cancerous prostate tissue compared with that in normal tissue. As cancerous tissue is composed of predominantly luminal epithelial cells, the possibility that loss of IGFBP-rP1 mRNA expression in cancer tissue is due to lack of IGFBP-rP1 expression in luminal epithelial cells has to be taken into consideration. However, our *in situ* data show a clear increase in grain intensity in the luminal epithelial cells, suggesting IGFBP-rP1 production by luminal cells. Loss of IGFBP-rP1 expression is therefore associated with progression of malignancy.

The decrease in IGFBP-rP1 expression in malignant prostate cells is consistent with the hypothesis that IGFBP-rP1 may have tumor-suppressive activity, although the specific role(s) of IGFBP-rP1 has yet to be determined. A clue to one role that IGFBP-rP1 may play in cell growth came from our studies using M12 cells (45), a tumorigenic subline of P69 cells. In these studies, IGFBP-rP1 appears to have proapoptotic activities, as overexpression of IGFBP-rP1, generated by stable transfection of M12 cells with IGFBP-rP1 cDNA, dramatically decreased the growth rate of the transfected cells and concomitantly increased the sensitivity of M12 cells to apoptotic agents such as 6-hydroxyurea (C. Tomasini-Sprenger, submitted). Thus, it is possible that in normal prostate cells, IGFBP-rP1 regulates cell growth, directly or indirectly, through apoptotic pathways, and loss of IGFBP-rP1 could, therefore, enhance abnormal cell growth.

IGFBP-rP1 mRNA was detected in all cell lines tested. However, IGFBP-rP1 protein was detectable in CM from primary cultures of prostate stromal and epithelial cells, P69 and M12, but not in CM from PC-3, DU145, or LNCaP prostate cancer cell lines. The inability to detect IGFBP-rP1 protein in CM from cancer cells does not rule out the possibility

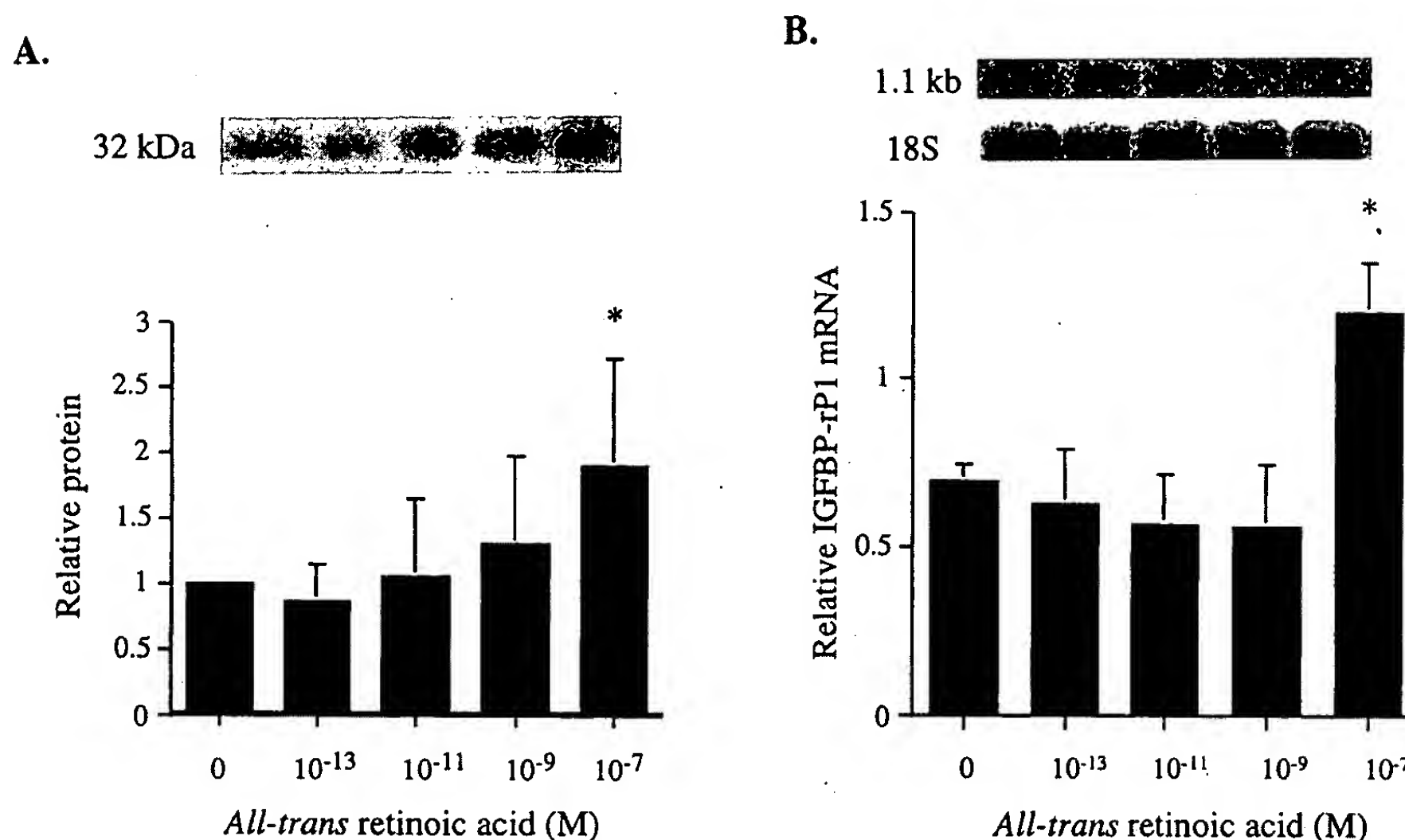


FIG. 5. Regulation of IGFBP-rP1 by all-*trans*-retinoic acid (RA) in P69 cells. P69 cells were treated with increasing doses of RA, and CM and total mRNA were collected after 24 h. A, Immunoblot analysis of IGFBP-rP1 expression. *Bar graphs* indicate relative amounts of IGFBP-rP1 protein (mean  $\pm$  SD), based on densitometric analysis of immunoblots. The *asterisk* indicates statistical significance ( $P < 0.05$ , by Mann-Whitney test). B, Northern blot analysis of IGFBP-rP1 expression. *Bar graphs* indicate relative concentrations of IGFBP-rP1 mRNA (mean  $\pm$  SD) based on densitometric analysis of Northern blots. The *asterisk* indicates statistical significance ( $P < 0.05$ , by Student's two-tailed unpaired *t* test).

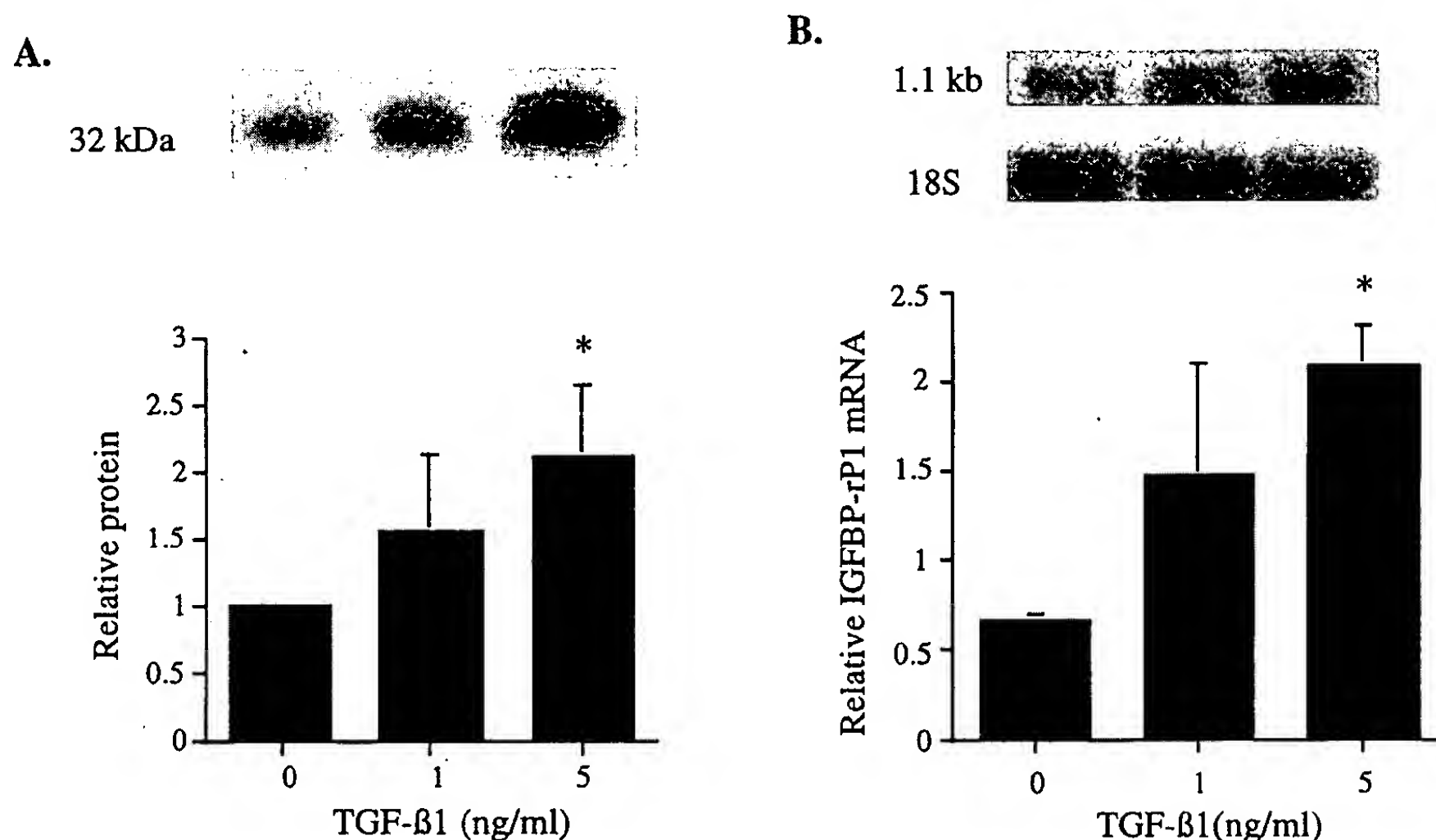


FIG. 6. Regulation of IGFBP-rP1 by TGF $\beta$ 1 in P69 cells. P69 cells were treated with increasing doses of TGF $\beta$ 1, and CM and total mRNA were collected after 24 h. A, Immunoblot analysis of IGFBP-rP1 expression. *Bar graphs* indicate relative amounts of IGFBP-rP1 protein (mean  $\pm$  SD), based on densitometric analysis of immunoblots. The *asterisk* indicates statistical significance ( $P < 0.05$ , by Mann-Whitney test). B, Northern blot analysis of IGFBP-rP1 expression. *Bar graphs* indicate relative concentrations of IGFBP-rP1 mRNA (mean  $\pm$  SD) based on densitometric analysis of Northern blots. The *asterisk* indicates statistical significance ( $P < 0.05$ , by Student's two-tailed unpaired *t* test).

that a very low concentration of IGFBP-rP1 is present in the CM. These results are consistent with decreased expression of IGFBP-rP1 in epithelial cells of malignant prostate. Further evidence that decreased IGFBP-rP1 expression occurs with tumorigenesis can be found in the reduced expression of

IGFBP-rP1 mRNA and protein between the P69 and M12 cells. The P69 cells are poorly tumorigenic, whereas the subline M12 is highly tumorigenic and metastatic (43, 45, 46). P69 cells express much higher levels of IGFBP-rP1 mRNA and protein than M12 cells. As these cells are linearly related, this



is evidence for a decrease in IGFBP-rP1 expression during transformation.

Interestingly, primary cultures of stromal cells expressed extraordinary quantities of IGFBP-rP1 mRNA and protein. *In situ* hybridization experiments also indicate high levels of IGFBP-rP1 mRNA present in stromal cells in both normal and malignant prostate tissue. The reason for the high expression of IGFBP-rP1 by stromal cells has yet to be determined, but one possibility is that the secreted IGFBP-rP1 protein from stromal cells may act as a paracrine regulator of normal epithelial cell growth.

Regulation of IGFBP-rP1 expression by IGF-I, RA, and TGF $\beta$  was also examined in all cell lines. Only in P69 cells was IGFBP-rP1 regulation detectable; there was no obvious regulation of IGFBP-rP1 protein in normal prostate epithelial or stromal cells, M12 cells, or from prostate cancer cell lines, PC-3, DU145, and LNCaP (data not shown). Consistent with observations reported for normal mammary epithelial cells (38), IGFBP-rP1 was also modestly up-regulated 2-fold by RA at both the mRNA and protein levels in the immortalized epithelial P69 cells, although the same regulation was not seen in primary cultures of epithelial cells. It should be noted that in normal mammary epithelial cells, IGFBP-rP1 regulation was only detectable in early and midpassages of the cells, and regulation was lost upon further passaging of the cells. This suggests a narrow window of time in which RA appears to be capable of regulating IGFBP-rP1 expression and could be easily missed.

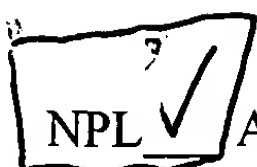
IGFBP-rP1 expression in P69 cells is also up-regulated by TGF $\beta$ 1. Both RA and TGF $\beta$ 1 are known inhibitors of epithelial cell growth. In breast cancer cells, it has been clearly demonstrated that the growth inhibitory action of TGF $\beta$ 1 and RA is mediated in part by the up-regulation of IGFBP-3 protein (47, 48), which has antiproliferative activity (15, 35). It is, therefore, conceivable that in P69 cells, the actions of TGF $\beta$ 1 and RA may be partially mediated by IGFBP-rP1. TGF $\beta$ 1 also specifically up-regulated IGFBP-3 in P69 cells; the antiproliferative function of IGFBP-3 in these cells cannot, therefore, be excluded. The roles of IGFBP-rP1 and IGFBP-3 in mediating TGF $\beta$ 1 and RA actions are currently under investigation. Interestingly, IGF-I, which is mitogenic for P69 cell growth (46), up-regulates IGFBP-rP1 as well as the other IGFBPs detectable in P69 cells. It is not clear what roles IGFBPs and IGFBP-rP1 play in IGF-I-stimulated growth.

In this report we have characterized the expression of IGFBP-rP1, a member of the IGFBP superfamily (20), in prostate cells. The observations that IGFBP-rP1 expression is significantly diminished with tumorigenesis and that expression is regulated in nonmalignant epithelial cells indicate the importance of IGFBP-rP1 in normal prostatic cell growth. The findings further support a potential antiproliferative effect of IGFBP-rP1 in the prostate, either by proapoptotic means or other mechanisms.

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1) Survey of gene amplifications during prostate cancer progression by high-throughput fluorescence in situ hybridization on tissue microarrays

AUTHOR: Bubendorf Lukas; Kononen Juha; Koivisto Pasi; Schraml Peter; Moch Holger; Gasser Thomas C; Willi Niels; Mihatsch Michael J; Sauter Guido; Kallioniemi Olli-P (Reprint)

AUTHOR ADDRESS: Cancer Genet. Branch, Natl. Human Genome Res. Inst., NIH, 49 Convent Dr., MSC 4470, Room 4A24, Bethesda, MD 20892-4470, USA\*\*USA

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2) \*\*\*Characterization of insulin-like growth factor-binding protein-related protein-1 in prostate cells.

Hwa V; Tomasini-Sprenger C; Bermejo A L; Rosenfeld R G; Plymate S R  
Department of Pediatrics, Oregon Health Sciences University, Portland 97201, USA.

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3) \*\*\*Glycotyping of prostate specific antigen.

Prakash S; Robbins P W

Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139, USA.

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Thank you

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# Survey of Gene Amplifications during Prostate Cancer Progression by High-Throughput Fluorescence *in Situ* Hybridization on Tissue Microarrays<sup>1</sup>

Lukas Bubendorf, Juha Kononen, Pasi Koivisto, Peter Schraml, Holger Moch, Thomas C. Gasser, Niels Willi, Michael J. Mihatsch, Guido Sauter, and Olli-P. Kallioniemi<sup>2</sup>

Laboratory of Cancer Genetics, National Human Genome Research Institute, NIH [L. B., J. K., O-P. K.], Bethesda, Maryland 20892-4470; Laboratory of Cancer Genetics, Tampere University Hospital, 33521 Tampere, Finland [P. K.]; and Institute for Pathology [P. S., H. M., N. W., M. J. M., G. S.] and Urologic Clinics [T. C. G.], University of Basel, CH-4003 Basel, Switzerland

## Abstract

Prostate cancer development and progression is driven by the accumulation of genetic changes, the nature of which remains incompletely understood. To facilitate high-throughput analysis of molecular events taking place in primary, recurrent, and metastatic prostate cancer, we constructed a tissue microarray containing small 0.6-mm cylindrical samples acquired from 371 formalin-fixed blocks, including benign prostatic hyperplasia ( $n = 32$ ) and primary tumors ( $n = 223$ ), as well as both locally recurrent tumors ( $n = 54$ ) and metastases ( $n = 62$ ) from patients with hormone-refractory disease. Fluorescence *in situ* hybridization (FISH) was applied to the analysis of consecutive tissue microarray sections with probes for five different genes. High-level ( $\geq 3X$ ) amplifications were very rare ( $< 2\%$ ) in primary prostate cancers. However, in metastases from patients with hormone-refractory disease, amplification of the androgen receptor gene was seen in 22%, *MYC* in 11%, and *Cyclin-D1* in 5% of the cases. In specimens from locally recurrent tumors, the corresponding percentages were 23, 4, and 8%. *ERBB2* and *NMYC* amplifications were never detected at any stage of prostate cancer progression. In conclusion, FISH to tissue microarray sections enables high-throughput analysis of genetic alterations contributing to cancer development and progression. Our results implicate a role for amplification of androgen receptor in hormonal therapy failure and that of *MYC* in the metastatic progression of human prostate cancer.

## Introduction

Prostate cancer is the most frequent cancer among men in industrialized countries and the second leading cause of cancer-related death (1). Given the substantial variability in the clinical behavior of prostate cancer, it would be important to better understand the biological basis of tumor development and progression, to develop markers for assessing prognosis or prediction of therapy outcome, as well as to identify targets for the development of novel therapies (2). The number of potential cancer-related genes and genetic alterations is increasing rapidly. The evaluation of the clinical utility of each of these genes would require multiple consecutive experiments with hundreds of tumors. This would be both time-consuming as well as impractical for more than a handful of genes.

We recently developed a novel tissue microarray ("tissue chip") technology (3) for rapid molecular profiling of large numbers of cancers in a single experiment. Tissue microarrays are constructed by bringing minute cylindrical tissue samples (diameter, 0.6 mm) from hundreds of different tumors into a single paraffin block. Five- $\mu$ m

sections from these tissue microarray blocks can then be applied in the analysis of copy number or expression of multiple genes by DNA and RNA *in situ* hybridization or by immunohistochemistry. Here, we constructed a tissue microarray containing samples from different stages of human prostate cancer progression to survey genetic alterations that may contribute to hormone refractory and metastatic disease. We decided to investigate the role of gene amplifications, because these alterations have been implicated in the progression of many tumor types. Most previous studies have found few if any gene amplifications in prostate cancer, but the majority of these have been based on relatively small materials or evaluated only a single gene (4-9). The comparison of data from different studies is also difficult, because a large variety of different techniques have been used, including Southern blot, slot blot, quantitative PCR, and FISH.<sup>3</sup> For example, substantially discordant results have been published on the role of *ERBB2* oncogene with the reported amplification frequencies ranging from 0 to 44% (4, 10-12). The two gene amplifications that have been studied in more detail include the *AR* and *MYC* oncogene amplifications reported in hormone-refractory or metastatic tumors, respectively (7-9). In this study, we constructed a tissue microarray containing 339 tumor specimens from different stages of prostate cancer progression and assayed five different gene amplifications (*AR*, *MYC*, *ERBB2*, *CCND1*, and *NMYC*) by FISH to consecutive formalin-fixed tissue microarray sections. The aim was to obtain a comprehensive survey of gene amplifications in different stages of prostate cancer progression, including specimens from distant metastases.

## Materials and Methods

**Prostate Cancer Tissue Microarray.** Formalin-fixed and paraffin-embedded tumor and control specimens were from the archives of the Institutes for Pathology, University of Basel and the Tampere University Hospital. All tumors and controls were reviewed by one pathologist (L. B.). The least differentiated tumor area was selected to be sampled for the tissue microarray. The specimens that were interpretable for at least one FISH probe included: (a) transurethral resections from 32 patients with BPH to be used as controls; (b) 223 primary tumors, including 64 cancers incidentally detected in transurethral resections for BPH; (c) 145 clinically localized cancers from radical prostatectomies, and 14 transurethral resections from patients with primary, locally extensive disease (13); (d) 54 local recurrences after hormonal therapy failure including 31 transurethral resections from living patients and 23 specimens obtained from autopsies; and (e) 62 metastases collected at the autopsies from 47 patients who had undergone androgen deprivation by orchiectomy and had subsequently died of end-stage metastatic prostate cancer. Metastatic tissue was sampled from pelvic lymph nodes ( $n = 8$ ), lung ( $n = 21$ ), liver ( $n = 16$ ), pleura ( $n = 5$ ), adrenal gland ( $n = 5$ ), kidney ( $n = 2$ ), mediastinal lymph nodes ( $n = 1$ ), peritoneum ( $n = 1$ ), stomach ( $n = 1$ ), and ureter ( $n = 1$ ). In 23 autopsies, material was available from both the primary and from the metastatic site. More than one sample per tumor specimen was arrayed in 44 of the

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<sup>2</sup> To whom requests for reprints should be addressed, at Cancer Genetics Branch, National Human Genome Research Institute, NIH, 49 Convent Drive, MSC 4470, Room 4A24, Bethesda, MD 20892-4470. Phone: (301) 435-2896; Fax: (301) 402-7957; E-mail: okalli@nhgri.nih.gov.

<sup>3</sup> The abbreviations used are: FISH, fluorescence *in situ* hybridization; AR, androgen receptor; CCND1, Cyclin-D1; BPH, benign prostatic hyperplasia.



339 cases. A tumor was considered amplified if at least one sample had amplification.

The original array also included 48 pathologically representative samples that consistently failed in the analysis of sections with all FISH probes. The number of samples evaluable with the different probes was variable because: (a) the hybridization efficiency of the probes was slightly different (see "Results"); (b) some samples on the array were occasionally lost during the sectioning or FISH procedure; and (c) some tumors were only representative on the surface of the block, and the morphology changed as more sections were cut.

**Construction and Sectioning of Tissue Microarrays.** The prostate tissue microarray was constructed as described previously (3). Briefly, a tissue arraying instrument (Beecher Instruments, Silver Spring, MD) was used to create holes in a recipient paraffin block and to acquire tissue cores from the donor block by a thin-walled needle with an inner diameter of 0.6 mm, held in an X-Y precision guide. The cylindrical sample was retrieved from the selected region in the donor and extruded directly into the recipient block with defined array coordinates. A solid steel wire, closely fit in the tube, was used to transfer the tissue cores into the recipient block. After the construction of the array block, multiple 5- $\mu$ m sections were cut with a microtome using an adhesive-coated tape sectioning system (Instrumedics, Hackensack, NJ). H&E-stained sections were used for histological verification of tumor tissue on the arrayed samples.

**FISH to Formalin-fixed Tissue Microarray Sections.** Two-color FISH to sections of the formalin-fixed samples on the tissue microarray was performed using Spectrum Orange-labeled AR, MYC, ERBB2, and CCND1 probes with corresponding FITC-labeled centromeric probes (Vysis, Downer's Grove, IL). In addition, one-color FISH was done with Spectrum Orange-labeled NMYC probe (Vysis). The hybridization was performed according to the manufacturer's instructions. The following tissue treatment protocol was developed to allow formalin-fixed tumors on the array to be reliably analyzed by FISH. The slides of the prostate microarray were first deparaffinized, immersed in 0.2 N HCl, incubated in 1 M sodium thiocyanate solution at 80°C for 30 min, and immersed in a protease solution (0.5 mg/ml in 0.9% NaCl; Vysis) for 10 min at 37°C. The slides were then postfixed in 10% buffered formalin for 10 min, air dried, denatured for 5 min at 73°C in 70% formamide/2 $\times$  SSC (SSC is 0.3 M sodium chloride and 0.03 M sodium citrate) solution and dehydrated in 70, 80, and 100% ethanol, followed by proteinase K (4  $\mu$ g/ml PBS; Life Technologies, Inc., Rockville, MD) treatment for 7 min at 37°C. The slides were then dehydrated and hybridized. The hybridization mixture contained 3  $\mu$ l of each of the probes and Cot1-DNA (1 mg/ml; Life Technologies, Inc.) in a hybridization mixture. After overnight hybridization at 37°C in a humid chamber, slides were washed and counterstained with 0.2  $\mu$ M DAPI. FISH signals were scored with a Zeiss fluorescence microscope (Jena, Germany) equipped with a double-band pass filter using  $\times 40$ - $\times 100$  objectives. The relative number of gene signals in relation to the centromeric signals was evaluated by visual analysis of the hybridization signals. Criteria for gene amplification were: tight clusters of signals in multiple cells or at least three times more test probe signals than centromeric signals per cell in  $>10\%$  of the tumor cells. Test:control signal ratios in the range between 1 and 3 were regarded as low-level gains and were not scored as evidence of specific gene amplification. Evidence for amplification of NMYC without reference probe was considered in the case of tight clusters of gene signals or  $>5$  signals in at least 10% of the tumor cells.

## Results

**FISH Analysis of Formalin-fixed Tissue Microarray Sections.** After optimizing pretreatment of tissue microarray sections, the quality of FISH results from formalin-fixed tumors was in most instances comparable with that obtained with ethanol-fixed frozen tissues used in our previous studies (Fig. 1). The hybridization efficiency of the probes could be estimated from the analysis of BPH tissue samples that were present on the same tissue microarray. High-quality hybridization signals with both centromeric, and gene-specific probes were obtained in 96% of the BPH samples for chromosome X/AR gene, 84% for chromosome 8/MYC, 81% for chromosome 17/ERBB2, and 83% for chromosome 11/CCND1. In the evaluable BPH samples, the average percentage of epithelial cells with two signals for autosomal probes was  $\sim 75\%$ , with  $\sim 20\%$  showing one signal and  $\sim 5\%$  no signals. The percentage of cells with one or zero signals is probably mostly attributable to the truncation of nuclei with sectioning (14). In

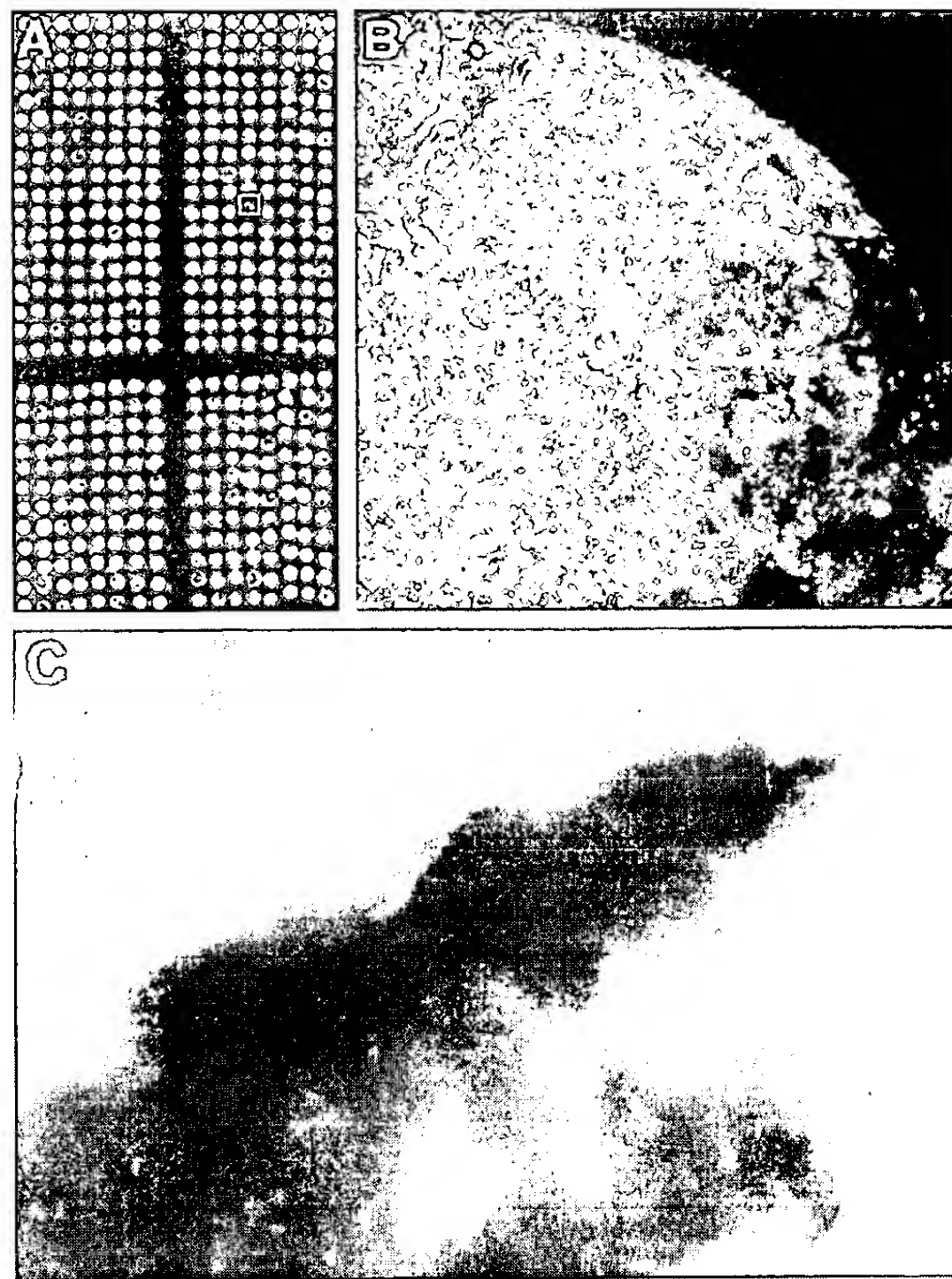


Fig. 1. Detection of AR amplifications in prostate cancer by FISH on sections of a prostate tissue microarray from formalin-fixed tissue specimens. A, overview of a tissue microarray section containing hundreds of different tumor samples ( $\varnothing$  0.6 mm, each).  $\times 3$ . B and C, AR amplification with many clustered AR gene signals (red) and a few centromere X reference signals (green). B,  $\times 200$ ; C,  $\times 1000$ .

the punched samples from biopsy cancer specimens, AR, MYC, ERBB2, and CCND1 FISH data could be obtained from 92, 78, 82, and 86% of the cases, respectively. The success rate of FISH was lower in punches from autopsy tumors (44–58%). Amplifications were only scored to be present when the copy number of the test probe exceeded that of the chromosome-specific centromere reference probe by  $\geq 3$ -fold in 10% or more of the tumor cells. This criterion was chosen, because low-level amplification is likely to be less relevant, and because locus-specific probes often display slightly higher copy numbers than centromeric probes, due to signal splitting or the presence of G<sub>2</sub>-M-phase cells.

**AR.** FISH with the AR probe revealed amplification in 23.4% of the 47 evaluable hormone-refractory local recurrences (Fig. 2). Amplification was seen equally often (22.0%) in 59 metastases of hormone-refractory tumors. The strong association between AR amplification and hormone-refractory prostate cancer is evident from the fact that only 2 of the 205 evaluable primary tumors (1%) and none of the 32 BPH controls showed any AR amplification. The two exceptions included a patient with locally advanced and metastatic prostate cancer and another patient with clinically localized disease. Whereas the patient records did not mention hormonal therapy, prior exposure of these patients to such therapy cannot definitively be ruled out. Paired tumors from the primary site of the cancer and from a distant metastasis of 17 patients were successfully analyzed for AR amplification. In 11 of these patients, no AR amplification could be seen at either site. Of the six remaining patients, three patients showed amplification in the local tumor mass, as well as in the distant

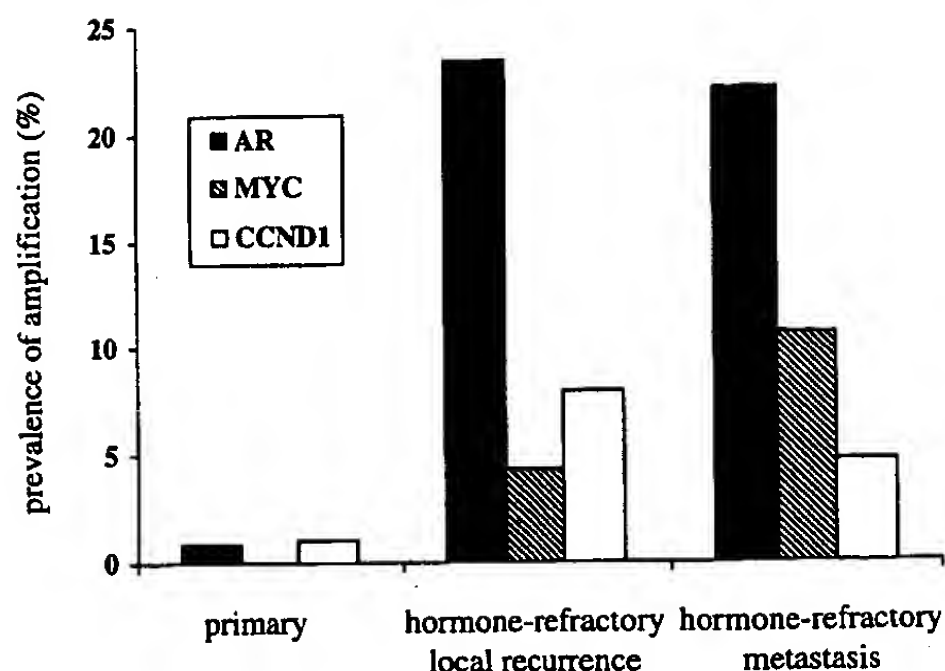


Fig. 2. Prevalence of oncogene amplifications during the progression of prostate cancer by FISH on prostate tissue microarray ( $n = 371$  specimens). There were no amplifications of ERBB2 and NMYC.

metastases. In two cases, amplification was only found in the sample from the primary site, whereas in another case, only the distant metastasis showed amplification.

**MYC.** High-level MYC amplifications were found in 5 of 47 evaluable metastatic deposits (10.6%), in 2 of the 47 local recurrences (4.3%, both from cancers that also had metastases), but in none of the 168 evaluable primary cancers or 31 BPH controls (Fig. 2). There was a significant association between AR and MYC amplifications. MYC was amplified in 12.5% of 24 evaluable tumors with AR amplifications but only in 1.8% of 219 tumors without AR amplifications ( $P = 0.003$ , contingency table analysis). AR was independently amplified in 21 tumors, whereas only 4 tumors had MYC amplification, but no AR amplification.

**CCND1, ERBB2, and NMYC.** CCND1 amplifications were found in 2 (1.2%) of the 172 evaluable primary tumors, in 3 (7.9%) of 38 local recurrences, and in 2 (4.7%) of the 43 metastases. CCND1 amplification appeared independent from AR or MYC amplification with only two of seven and one of seven CCND1-amplified tumors showing also AR or MYC amplification, respectively. There were no ERBB2 amplifications among any of the 262 evaluable tumors or 31 BPH controls. Finally, a subset of the tumors was analyzed with the NMYC probe in a single-color FISH analysis. Of the 164 tumors available, none showed evidence of amplification.

## Discussion

Development and progression of prostate cancer to lethal, hormone-refractory, and metastatic disease is believed to be driven by multiple genetic alterations, the nature and sequence of which have remained poorly understood. For this study, a tumor tissue microarray was constructed that allowed us to investigate the pattern of amplifications of multiple genes in samples representing the entire spectrum of prostate cancer progression, including distant metastases. The advantage of the tissue microarray strategy is that it facilitates standardized analysis of multiple genes in the same tumors, even in the same specific tumor sites using the same technology, same kind of probes, and similar interpretation criteria. In just five FISH experiments, we were able to screen a material of 371 specimens with five gene-specific probes, resulting in a total of >1400 evaluable FISH results. The ability to achieve reliable detection of gene amplifications from formalin-fixed tissues substantially extends the range of possible applications for the tissue microarray technology (3) by enabling studies of tumor progression using archived surgical or even autopsy specimens. In this study, we focused on high-level gene amplifications that are relatively straightforward to detect and score visually. However, we expect

that in the future, automated digital image scanning and spot counting of entire tissue microarray slides will become possible and will increase reproducibility and facilitate detection of other alterations, such as deletions or low-level gene copy number gains.

A possible limitation of the tissue microarray technology is that the minute tissue samples acquired from the original tissues may not always be representative of the entire tumor, in light of the intratumor heterogeneity characteristic to most cancers. The frequency of involvement of the different genes in our study is therefore likely to be an underestimate, although the comparison of the present results with the previous literature suggests that this problem is not as substantial as one would expect (see below). The effect of this sampling bias is also primarily reflected in the analysis of absolute frequencies of genetic alterations in a given tumor type. The comparisons between similarly acquired specimens from different stages of tumor progression placed on the same tissue microarray should be less problematic. Also, comparisons of the frequencies of involvement of specific genes with those of other genes evaluated from consecutive tissue microarray experiments should suffer little if at all of sampling biases. Moreover, it is very likely that "punching" from multiple sites from each original tumor can significantly reduce the sampling problem. Nevertheless, at the moment, one should consider the tumor tissue microarray technology as a rapid, high-throughput survey method to pinpoint the biologically most prevalent or clinically most promising genes and molecular markers for detailed studies with conventional tissue specimens.

Gene amplifications have been reported to be more infrequent in prostate cancer than in many other carcinomas. According to our results, this is indeed the case for primary prostate cancers, where high-level amplifications of all of the tested loci were rare (<2%). However, in samples from hormone-refractory local recurrences or metastatic deposits, the amplification frequencies were substantially more common for three of the five genes evaluated. This is in agreement with the hypothesis that accumulation of multiple genetic changes, perhaps as a result of genetic instability, is associated with prostate cancer progression (15–17).

In our previous studies, up to 30% of patients failing hormonal treatment were found to have AR amplification (8, 9). In these studies, only tissues from the locally recurrent tumors were available. The present tumor tissue microarray analyses of end-stage metastatic patients indicated that AR amplification is equally common in the distant metastatic deposits. Studies of the molecular genetic changes in the metastatic specimens are important, because the distant metastatic sites are primarily responsible for the clinical outcome, and represent the primary targets of systemic therapies (18). In one-half of the patients with end-stage hormone-refractory disease associated with AR amplification, amplified cells were present in all sites sampled, both in the locally recurrent tumors, as well as in distant metastases. In the remaining patients, AR amplification was only present in either the local site or in the metastases. Because most of the patients have metastatic disease already before androgen deprivation therapy is initiated, it is likely that the different sites of cancer in the same patient may sometimes respond to hormonal treatment in a unique manner. Our results suggest that tumor progression to hormone refractory cancer develops via different molecular mechanisms. This heterogeneity may also explain why any therapy against metastatic, hormone-refractory prostate cancer often tends to be ineffective.

MYC amplifications were most often found in the distant metastatic deposits sampled at autopsy. This 11% prevalence is somewhat lower than the previously reported 21% frequency of MYC involvement in prostate cancer metastases to pelvic lymph nodes (7). It is possible that the sampling from only one distinct region of each tumor may have led to an underestimation of the true prevalence of gene amplification in our study. However, in the study by Jenkins *et al.* (7), also a less stringent



criterion ( $>2X$ ) for amplification was used than in our study ( $\geq 3X$ ). Because low-level increases of copy numbers of the long arm of chromosome 8 are so common (15, 16, 19), it is possible that the more stringent cutoff is more appropriate to identify cases, where specific amplifications of the *MYC* oncogene region take place. One should note, however, that the finding of *MYC* amplification by FISH does not prove that *MYC* is the target gene of the amplification at 8q24.

Our study provides evidence for *CCND1* gene amplification in human prostate cancer *in vivo*. The amplification frequency was low ( $\sim 1\%$ ) in primary prostate cancer, which may explain why it has not been reported previously. In contrast, 4.7–7.9% of the hormone-refractory and metastatic samples had *CCND1* amplification. Further studies are required to evaluate the significance of this amplification for prostate cancer progression. Interestingly, *CCND1* amplification often appeared to take place independently of *AR* and *MYC* amplifications.

One group has previously suggested that *ERBB2* amplification is a frequent genetic alteration and has prognostic importance in prostate cancer (11, 12). However, other investigators have failed to detect *ERBB2* amplifications in prostate cancer (4, 10). Similarly, we did not detect any *ERBB2* amplifications at any stage of cancer progression, including end-stage autopsy tumors. It is likely that the high prevalence of *ERBB2* amplifications reported in the study of Ross *et al.* (11, 12) was due to a less stringent definition of gene amplification and the lack of a chromosome-specific reference probe to exclude the influence of aneuploidy. On the basis of our comparative analysis of *AR*, *MYC*, *CCND1*, and *NMYC* amplifications in the identical tumor samples, using the same FISH methodology and interpretation criteria, we do not expect either *ERBB2* or *NMYC* amplifications to play any significant roles in the *in vivo* progression of human prostate cancer.

Many symptomatic prostate cancers become both hormone-refractory and metastatic, and it is very difficult to distinguish between these two clinical features or the molecular mechanisms that contribute specifically to either one of these processes. Taking our present results together with previous information (7–9), one can formulate a hypothesis that *AR* amplification is more closely associated with the development of hormone-refractory cell growth, whereas *MYC* amplification is associated with metastatic progression. Our results suggest that the most common gene amplification in prostate cancers is that of the *AR* gene, which is usually amplified independently of both *MYC* and *CCND1*. *AR* has been shown to be amplified in locally recurrent tumors from patients who do not have evidence of distant metastases (8), whereas *MYC* amplifications have been associated with metastatic progression (7). Indeed, in our present study, *MYC* amplifications were more common in the distant metastases (11%) than in the locally recurrent tissues (4%; both two patients with end-stage metastatic cancers), whereas *AR* amplifications were equally common at both anatomical sites (22 and 23%, respectively). This suggests that *AR* is conferring an advantage for hormone-refractory growth and not metastatic dissemination, whereas the reverse may be true for *MYC*. *MYC*-amplified tumors may also often contain *AR* amplification. One could speculate that the selection force responsible for the development of *AR* amplification makes it necessary for the cells to overcome the checkpoints that prohibit gene amplification in normal cells. This would lead to amplification of other genes such as *MYC*.

In conclusion, these results illustrate that the tissue microarray technology is a powerful tool for the molecular profiling of large numbers of tumors representing the entire disease spectrum of human prostate cancer progression *in vivo*. This high-throughput, tissue microarray-based screening by FISH identified distinct patterns and interrelationships between the different gene amplifications, leading to hypotheses that can now be tested in future studies of large specimens, or by more extensive sampling from each tumor site: (a) the present results suggest that *AR* gene is the most frequent target,

and often the first target, selected for amplification during prostate cancer progression; (b) in contrast to *AR*, amplifications of the *MYC* oncogene may be primarily associated with metastatic dissemination; and (c) prostate cancers occasionally also amplify the *CCND1* gene, whereas *ERBB2* and *NMYC* amplifications are unlikely to play a significant role at any stage of the progression of prostate cancer. Additional studies with the "tissue chip" approach may be helpful to generate a more comprehensive model of the genetic and molecular steps associated with prostate cancer progression, as well as to help the translation of such biological findings to clinical applications.

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